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*Topical Review*

# Membrane Transport Properties of L-2,4-Diaminobutyrate Revisited*

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**Summary.** We explore here the special structural features of certain diamino acid analogs which may account for their intense accumulation into tumor cells, first observed for the Ehrlich ascites tumor cell for in vitro suspensions. This accumulation, which ordinarily occurs mainly by system A for its dipolar substrates, is so intense for these tripolar diamino acids accompanied by the chloride ion as well as by displacement, especially of the cellular potassium ion, that the cells swell to several times their normal volume and osmotic destruction arises. These structural features receive our reconsideration here toward understanding the energization of amino acid transport into cells, also toward identifying among them possible superior $^{11}$C-labeled tracers for imaging tumors in situ by positron emission tomography (PET). The possibility of therapeutic, perhaps osmotic, destruction of inoperable terminal gliomas by topical application of such amino acids by microdialysis has also been considered in preliminary tests by one of us (G.R.) and his associates.

**Key Words** arginine · EcoR receptor · histidine · homoserine · lysine · oocytes · viral disease

We have been persuaded to keep open the question how the transmembrane transport of amino acids, when uphill, is energized, despite the clear role of their cotransport with $Na^+$. New evidence on that question, presented by a paper from the Parma laboratory (Dall'Asta et al., 1991), indicates for the human fibroblast, an untransformed cell with a highly concentrative system A, that the energy inherent in the transmembrane electrochemical potential of $Na^+$ over a range of 4 to 12 kJ per mol is closely correlated with and sufficient to account for the gradient by which MeAIB is accumulated. Nevertheless, at this unlikely time and without new personal results we seek to keep within our thinking possible roles for flows of hydrogen ions and of electrons, at least in the Ehrlich ascites tumor cell as studied by us.

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Let us remind you with Fig. 1 how the route of amino acid transport changes with pH (Christensen, 1968). Here we see that the main route of uptake of histidine changes with rising pH from the route typical for cationic amino acids to a route for dipolar amino acids, as indicated by the shift in sensitivity to system-specific inhibitory analogs. Figure 2 shows the dissociation behavior predicted for 2,4-diaminobutyrate (DAB) from titration curves and by analogies with other amino acids. From the comparison with the example of histidine one might expect DAB to be transported at pH 7.4 mainly as a cationic amino acid, presumably by a system we now call $y^+$. System $y^+$ proves, however, to be only a minor one for DAB transport in the Ehrlich ascites tumor cell, although one seen with special clarity in a cell lacking system A (read the penultimate paragraph of p.1503 by Christensen and Antonioli (1969)). That study preceded our satisfying ourselves (Christensen et al., 1969) that certain dipolar amino acids, including DAB, not only inhibit but really migrate and exchange along with $Na^+$ via the otherwise $Na^+$-independent system $y^+$. In the Ehrlich cell system A predominated in DAB transport, most of its uptake unexpectedly inhibited both by 2-aminoisobutyrate (AIB) and by its N-methyl derivative, MeAIB, as shown in Fig. 3 by a Dixon plot for that inhibition by MeAIB (Christensen & Liang, 1966). Other characteristics of this uptake are unambiguously those for system A for dipolar amino acids, including $Na^+$ dependence and its pH sensitivity, even though DAB is characteristically tripolar. Since its pK'2 is only 8.24, a minor transport via system A would *a priori* not be surprising, a minor proportion of this amino acid being indeed dipolar rather than cationic at neutrality.

Unexpectedly, despite its uniquely high total accumulation, DAB doesn't show high affinity for any known transport system of the Ehrlich cell. For system A, its $K_m$ remarkably is as high as 60 mM

(Christensen & Liang, 1966)! Now 60 mM at first thought may not seem an unreasonable $K_m$ for DAB, considering that (Fig. 2) only about one-tenth of the DAB should at neutrality be in a dipolar form, and hence for the aggregate concentration of DAB a $K_m$ ten times an ordinary value of 5 or 6 mM for a system A substrate is not surprising (although note the peculiar exception of MeAIB; Inui & Christensen, 1966, Fig. 13). But on further thought even that high estimate proves illogically low since most of the dipolar DAB in free solution will be in the omega dipolar form (Fig. 2), normally not expected to react with ordinary neutral amino acid transport systems. A titration of DAB by proton magnetic resonance (Fig. 4) confirms that the omega zwitterion is, as expected, the one formed mainly during the second (pK'2) stage of its ordinary acid-base titration (Christensen, 1984). So DAB might for the moment be expected to react scarcely at all with system A!

Figure 5 shows our 1952 finding (Christensen et al., 1952) that an enormous uptake of DAB supplied at external concentrations of 30 mM and up continues for at least four hours. The thickening of the Gamble bar-diagram in Fig. 5 shows the resultant swelling of the Ehrlich cells, which continued until osmotic cytolysis began. Hence the actual transfer of ions was much larger than one might judge from looking only at the height of these bars. The algebraically summated shift in ions, considering uptake of the amino acid cation as its hydrochloride and its displacement of $Na^+$ and $K^+$, perhaps also of $Mg^{2+}$, was approximately such as to preserve a charge balance. When hypertonicity was maintained by including extra NaCl at 40 mM along with 40 mM amino acid hydrochloride in the suspending solution, cellular swelling was retarded, but the hypertonicity did not increase uptake of DAB during 4 hr (Christensen, et al. 1952). This behavior of the Ehrlich cell is consistent with a recent report that it can't volume-regulate following a hyperosmotic challenge (Levinson, 1991).

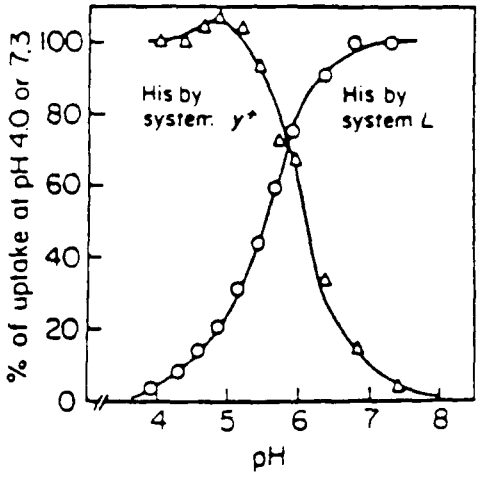


**Fig. 1.** Changes in the rate of uptake of L-histidine by a transport system for dipolar amino acids, shown by leucine inhibition (right) and separately (left) by one for tripolar cationic amino acids, shown by lysine inhibition (left). (Reproduced with permission from Im and Christensen, 1976.)

Given that the $K_m$ of this uptake of DAB is at least as high as 60 mM, how can we explain such enormous uptakes by the tumor cell? First, we should note that cancer cells show especially high system A activity, whereas in ordinary untransformed cells that system, largely in its repressed state, shows distinctly low reactivity in relation to other transport systems. Beyond its direct contribution, the activity of system A governs the energy made available to other transport systems by their exchange for other amino acids among those which it *per se* concentrates into the cell. Ordinarily system A is inconspicuous in comparison with system ASC; nevertheless, because of its special concentrative character it remains strategic for transport energization in general.

That contrast applies to the natural substrates of system A, such as alanine and glycine, and should apply to DAB. DAB presents, however, a very different picture. Beyond the effect of the vigorous reactivity of system A in this neoplastic cell, note that the very high $V_{max}$ value, certainly well above 30 mmol · $kg^{-1}$ · $min^{-1}$ DAB (Christensen & Liang, 1966), is the factor that accounts for its high uptake. Similar distortions in the kinetiç parameters apply, although to a lesser degree, for 1,2-diaminopropio-

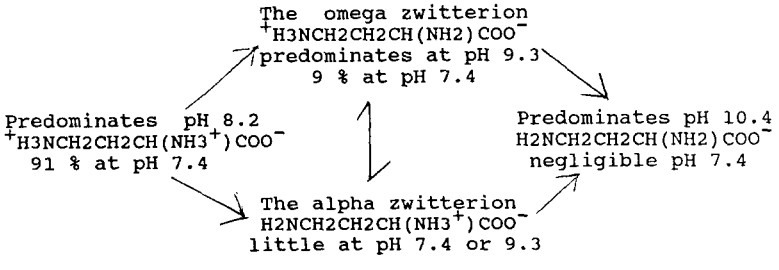


**Fig. 2.** Sequence of $H^+$ release, as predicted and then confirmed (Christensen, 1984) by proton magnetic resonance titration, given that of the two amino groups the one on the alpha carbon atom releases its hydrogen ion at lower pH values than does the one on the omega carbon. Hence formation of the omega zwitterion will predominate over the alpha, whether at pH 9.3 or 7.4.

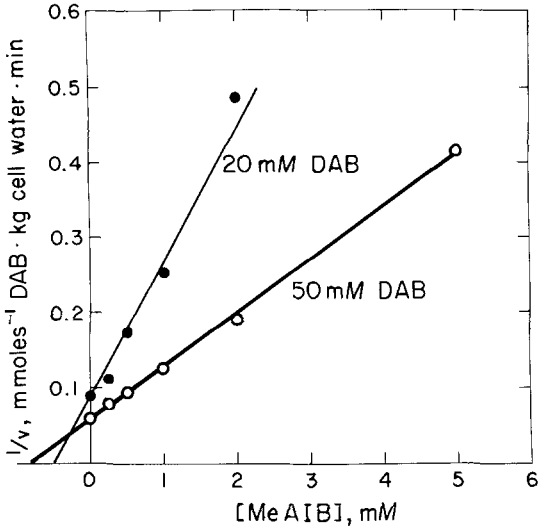


**Fig. 3.** Dixon plot of inhibition of L-2,3-diaminobutyrate (DAB) uptake by MeAIB during 2 min from Krebs-Ringer bicarbonate medium, pH 7.4 and 37°C. ($Na^+$) was 96 mM when DAB was 50 mM, and 135 mM when DAB was 20 mM. The two lines intersect at −0.3 mM. The higher sodium ion concentration in the latter plot has presumably contributed somewhat to its greater slope. (Reproduced with permission from Christensen and Liang, 1966.)

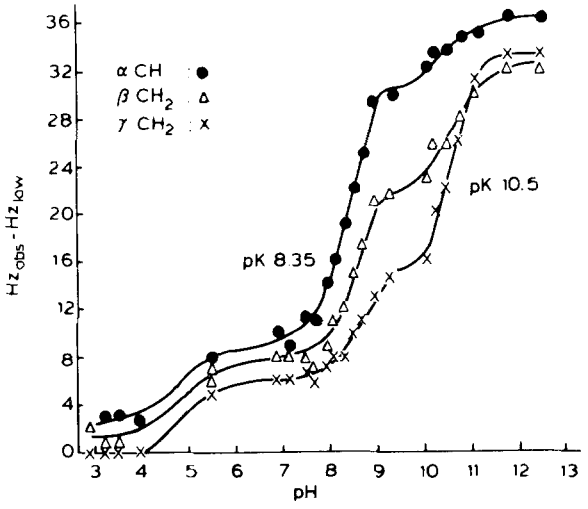


**Fig. 4.** Assignment of the sequence for DAB dissociation by proton magnetic spectroscopy. The pH values were obtained by plotting pH/Hz versus pH. The proton on the alpha carbon atom shows its largest chemical shift with a pK′ of 8.35, whereas that on the gamma carbon shows its largest shift at pK′ 10.5. This sequence is consistent with one following mainly the upper course pictured in Fig. 2, via the omega zwitterion. (Reproduced with permission from Fig. 3 in Christensen, 1984.)

nate. We believe this intense uptake of DAB results in part because the transport step applies to a dipolar ion, whereas overall it is mainly a cation that is accumulated; i.e., a proton will be transferred secondarily to reprotonate the transported dipolar ion, whether in its alpha or omega form, when it reaches the cellular environment, as required by the value of pK′2 for DAB. Hence overall we are dealing largely with the gradient of an organic cation, and therefore the transmembrane potential influences the value of the steady-state distribution that this amino acid approaches. The transmembrane potential is well recognized largely to determine the steady states reached for lysine or arginine accumulation by various cells.

This explanation for the strong accumulation of DAB seems by no means adequate, however, and one should not too readily dismiss other likely consequences of the chemical nature and geometry of this diamino acid. These must lead to its initial binding to the transporter in a dipolar form, despite two expectations: (i) that both amino groups should for the most part retain protons at neutrality in ordinary environments, and (ii) that the dipolar form initially acceptable to the transporter, namely the alpha-zwitterion, is present in very low concentration.

Clearly the predominant omega zwitterion should be unsuitable for transport by a system designed for transporting ordinary amino acids, as is nicely shown by the rejection of DAB as a system ASC substrate (Christensen, 1972). We conclude that system ASC sees and rejects DAB in the unacceptable omega dipolar form that DAB largely presents in free solution. Yet for system A, DAB is not only accepted, but undergoes accumulation to an extent that is unique in our experience. Hence the system A transporter, on encountering DAB first in its more abundant cationic and alpha-omega dipolar forms, must reject both and instead bind and stabilize DAB in its exceptional alpha-alpha dipolar form, which is clearly for ordinary amino acids the form selected for transport by system A.

We believe, however, that we do not complete a full and adequate explanation of the intense accumulation of DAB even when we conclude, logically enough, that the DAB cation is deprotonated unexpectedly at its omega amino group on acceptance for transport. To explain fully that intensity, we have urged that DAB is not only accepted as the alpha zwitterion but subsequently almost all of its dipolar form available for escape from the cell will be released in the form of the unacceptable omega zwitterion. Much more of the accumulated DAB will have been reprotonated to its ordinarily predominant free cationic form (Christensen et al., 1974). That striking difference between systems A and ASC in the rejection of DAB by the latter (Christensen, 1972), despite the near ideality of the highly analogous ASC substrate, homoserine, $HOCH_2CH_2CH(NH_3^+)COO^-$, must mean that ASC lacks the cata-

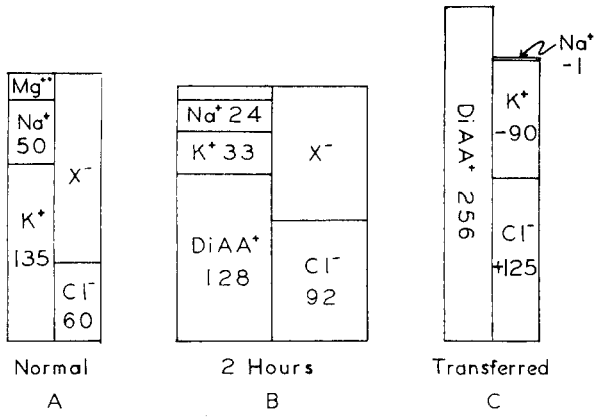


**Fig. 5.** Uptake of DAB by the Ehrlich ascites tumor cell. $DIAA^+$ = the DAB cation. (*A*) Typical concentrations of ions calculated for cell water 1 mEq per kg cell water. (*B*) After 2 hr in Krebs-Ringer bicarbonate medium containing initially 30 mM DAB as its hydrochloride. (*C*) Ions transferred to cells in mEq per kg original cell water. Swelling is indicated by the thickening of the bar *B*. (Reproduced with permission from Christensen et al., 1952.)

lytic feature that allows system A to manipulate the position of its single associated proton during DAB transport. That striking difference between systems A and ASC should perhaps not surprise us when we consider the flexibility of the latter in carrying either anionic, cationic or dipolar amino acids, according to its own state of protonation (Makowske & Christensen, 1982).

As an apparent parallel to the behavior of diaminobutyrate, for a number of other amino acid analogs to encounter unusually steep uphill transport by system L (Christensen et al., 1974) what seems to be needed is again a net cationic character for the amino acid (usually diamino). This cationic state is determined by a $pK_a'$ in the range 7 to 8.5, apparently low enough to make significant the degree of its catalytic deprotonation by the functioning transporter. This structural feature should, and not only for DAB, be closely examined for producing high amino acid labeling by PET. (This incidentally is not the only structural feature that secures strongly uphill system L transport; note the case (Ohsawa et al., 1980) of the bicyclic amino acid known as BCH, which provides us the least equivocal evidence that system L does indeed pump uphill the net of its amino acid substrates.) Given that $pK_2$ for the alpha amino group falls in that low range, we hold that such diamino acid analogs show membrane transport responsive to the $H^+$ concentration in the manner we have suggested elsewhere (Christensen et al., 1974) and will now recapitulate:

We have proposed that an intramembrane $H^+$ pumping first deprives DAB, serving as a transportable probe of intramembrane evironments, of its distal proton on acceptance of that cationic amino acid, and then appropriately restores the proton to DAB to activate its inward release across the membrane. We have proposed that such an intramembrane pumping of the hydrogen ion can cause this and similar amino acids to be intensely accumulated. This idea has been misunderstood by supposing that our exposition meant (Christensen et al., 1974) that sequential changes in the charge on the amino acid *per se* are what cause it to be "pumped." That might not provide an explanation beyond the obvious one arising from the preexisting transmembrane potential. Our proposition is different but nevertheless quite simple: Suppose, for a molecule about to continue its transmembrane course within the membrane, some part of the molecule is separated and independently pumped uphill over a finite interval, and that part then restored to yield the original molecule beyond an internal membrane barrier (Christensen et al., 1974). That pumping of a component of the molecule under these simple considerations will add to the chemical potential of the transported molecule and allow its release at a higher concentration. The challenging requirement of this proposal might be seen as the restriction of proton permeation through an effective barrier region of the system, i.e., the existence of at least two serial intramembrane compartments that allow little spontaneous interflow of protons. We fail to see how a $H^+$ gradient can ever drive uphill transport by symport unless the flow of the free $H^+$ is so restricted over a finite interval.

We have also found that the *trans* conformer of 1,4-diaminocyclohexanecarboxylic is much more weakly accumulated than its *cis* isomer (Christensen, 1972, Table II), showing that the steric positioning of the second amino group at the transporter receptor site can be a critical element for making these diamino acids subject to the mechanism facilitating their transmembrane accumulation. This finding shows that facilitated accumulation does not arise simply from an easier formation of a carbamate by the predictable reaction of bicarbonate with the amino group having a low pK, say below 8.5. Enhanced carbamate formation is suspected to cause neurotoxic effects of certain amino acid analogs, e.g. some lathyrogens (Max, 1991), and might contribute neurotoxic effects for DAB.

Energies of activation at any point may be high enough to prevent the vectorial effect of an intramembrane gradient established for the separated portion of the substrate, so its success will depend on that gradient being relatively high enough. For DAB the catalytic action encountered in a barrier region of the membrane is held first to selectively remove a hydrogen ion from the substrate to form the alpha zwitterion, and then, subsequent to cross-

ing a further barrier region, to restore to the substrate an equivalent $H^+$ at a higher chemical potential, bringing the transported molecule likewise to a higher chemical potential at a point of release. For DAB passage, the activation energy barriers may be pictured as kept low, first by presenting the receptor site in a form that initially causes the $H^+$ to be lost selectively from the omega amino group of the cationic substrate. It may be significant that the geometry of DAB in its deprotonated bound form allows the remaining proton to be shared to some degree between the two amino groups, so that its shift is facilitated. In an ultimate compartment a proton may then be restored to that system selectively at the alpha amino group, to regenerate the transported cationic substrate. This is, of course, the amino group that will be expected to bear a proton on a neutral amino acid at a point likely to have participated in linkage and release. Have we reached a stage at which we should examine whether the transmembrane asymmetry observable for the A transporter (Christensen, 1972) may have a similar basis in their rearrangement during transport of numerous helical protein domains proposed by Widdas and Baker (1991) for the human erythrocyte glucose transporter?

Evidence only remotely related (Christensen et al., 1974) suggested to us that energization of system A by inward flow of $Na^+$ into Ehrlich cells might not obligatorily or solely be applied directly to drive the uphill inflow of AIB and similar amino acids. Under tests of the effect of radical displacement of endogenous amino acids from the Ehrlich cell with AIB or similar analogs, under conditions described in detail, we found evidence that subsequent AIB uptake might be energized without any evident down-flow of $Na^+$, as though the presumably essential energy input were in this situation directed along a different pathway. Underestimates of the $Na^+$ electrochemical gradient should not adequately explain observation of a condition under which inappreciable amounts of $Na^+$ flow along with the test amino acid, unless energization occurs by another means. Although this provocative effect on the apparent course of transport System A energization has seemed significant to us, it has remained unattractive to others because we do not understand how it may be produced. Under these circumstances, it is perhaps not strange that our lab group has encountered a subtle and ill-defined condition under which the uncoupling effect of the AIB treatment was on an occasion not obtained by one associate but obtained independently by another. Yet the repeated and, we think, rather well-controlled demonstrations of the effect dissuade us from deemphasizing the suggestion, especially in light of the foregoing clues for energization of system A by $H^+$ flows, a mode of energization which does, however, prove rather more pressing for system L than for A (Christensen et al., 1974), and especially because new evidence from Englesberg's laboratory (Qian, Pastor-Anglada & Englesberg, 1991) indicates that regulation may be applied coordinately to system A and to the mRNA for the $\alpha$-1 subunit for the $Na^+$, $K^+$-ATPase gene of cultured wild-type Chinese hamster ovary cells. Significantly, this coordinated stimulation of system A transport did not depend on an increase of $Na^+$ levels. Although for the moment we encounter difficulties in grasping how the stimulated action of this ATPase can act to provide an alternative intermediation of the energy transfer for system A without an elevation of ($Na^+$), we need, we believe, to include also in our reflections a possible role for driving amino acid transport by an oxidation-reduction system resident in the plasma membrane of the eukaryotic cell, a concept long rejected but recently revived. We summarize elsewhere (Garcia-Sancho et al., 1977; Christensen, 1987) evidence for the relevance of redox energization to the present case.

One can note in the literature an occasional somewhat dubious tendency to expect the division of amino acid transport among systems in the brush borders of the intestinal and renal epithelia to fall into a pattern somewhat parallel to that among systems A/ACS/L, etc., seen frequently in plasma membranes. A recent report (Jessen et al., 1991) shows probably three different systems by which AIB uptake by proximal brush border vesicles from segments of the rabbit proximal renal tubule are driven by transmembrane gradients of $Na^+$ and/or $H^+$ under competition by dipolar amino acids as different as phenylalanine, alanine and glycine. Evidence for the presence of system A was neither included nor implied, although these results may justify care in supposing the modes of energization of AIB transport to be much the same from tissue to tissue. They also motivate us to continue to look more widely for quite a different broad-scope, $Na^+$-dependent amino acid transport systems of the sort seen in these brush-border membranes and in early murine conceptuses (Van Winkle, Christensen & Campione, 1985).

*A possible therapeutic utility for DAB?* Perhaps we should in justice mention that Irvine Fuhr as an NIH study-section official had urged in a correspondence related to an early application (Christensen et al., 1952) for research funding, to consider the possibility that DAB might be tested by topical infusion via the blood circulation to treat pancreatic cancer. In the meantime, occasional discouragement has been voiced for cancer treatment that includes

potentially mutagenic or carcinogenic agents. A clinical test of the novel therapeutic application of transport-driven osmotic action on a tumor has been initiated by one of us (Ronquist et al., 1992). Because of the inherent restrictions encountered in the clinical use of DAB when administered systemically as intravenous injections or infusions (e.g., via the carotid artery) Ronquist and his associates chose to make their thus far small clinical trial by administering DAB locally and continuously into malignant brain tumors via microdialysis. In this trial, the conditions of administration permitted reasonably high DAB concentrations to be sustained in the tumor region during a selected time interval. This behavior presumably is mainly due to system A since the irreversible cytolytic effect shown by 6 mM DAB on human glioma cells in vitro is largely prevented by the presence of 2 mM MeAIB. Similar effects on human glial cells required far higher respective concentrations of these two amino acids (Ronquist, Westermark & Hugosson, 1984). A comparison between rat hepatocytes and rat hepatoma cell lines illustrates the tendency of system A to dominate over system ASC after oncologic transformation (Handlogten et al., 1981). Concomitantly, as one would expect under dialysis, low molecular-weight compounds including physiological amino acids were undoubtedly removed from the tumor region, thus creating conditions favorable for the uptake of DAB into tumor cells. This detail is considered important since otherwise some of the physiological amino acids could act as competitive inhibitors of DAB transport into these cells.

Patients having inoperable malignant glioma (astrocytoma grade III and IV) were treated in this way for a total of 14 to 21 days without side effects assignable to DAB. Massive tumor necrosis occurred as judged by comparison of computed tomography before and after DAB treatment. The small number of patients with inoperable malignant glioma so far treated presented survival times about three times longer than the mean survival time for patients with malignant glioma receiving only radiotherapy. This trial is still in its initial phase; hence exact knowledge has not been obtained as to the optimal conditions for length of the administration period of DAB nor for DAB concentration, nor has knowledge been gained as to possible synergistic effects with other chemotherapeutic agents.

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## Note Added in Proof

Other instances continue to arise where transport competition occurs between analogs of unlike charge, e.g. between ornithine, on the one hand, and putrescine or its lower homologs, on the other hand, in the presence of 125 mM NaSCN (Medina, M.A., Uridiales, J.L., Nuñez De Castro, L., Sanchez-Jimenez, F., 1991. Diamines interfere with the transport of L-ornithine in Ehrlich-cell plasma membrane-vesicles. *Biochem. J.* **280:**825–827). Here DAB, the lower homolog of ornithine, may be expected to react like ornithine as it does in the rabbit reticulocyte (Christensen & Antonioli, 1969). HNC now speculates with Medina whether in this new competition $Na^+$ comigrates alone with the amino acid, or whether instead an anion, say $SCN^-$ or $Cl^-$, might comigrate alone with the diamine, thus also to produce a balanced flow of charge in the bidirectional exchange, a matter not yet ascertained. The circumstance that ornithine or DAB can be transported via system $y^+$ either as a dipolar or as a cationic amino acid allows the possibility of a heteroexchange between the cationic amino acid without $Na^+$ and its dipolar form with $Na^+$, leading to a net driving of that transport by $Na^+$ in one direction or the other, as determined by mass action. Might not similar directionality be produced by movement of a cosubstrate anion? Such possible asymmetries are bound to receive increased attention.